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Design, Synthesis and Evaluation of Antiinflammatory, Analgesic and Ulcerogenicity studies of Novel S-Substituted phenacyl-1,3,4-oxadiazole-2-thiol and Schiff bases of Diclofenac acid as Nonulcerogenic Derivatives

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Abstract—Diclofenac sodium is being used for its anti-inflammatory actions since 28 years, but as all the NSAIDs are suffering from the deadlier GI toxicities, diclofenac sodium is also not an exception to these toxicities. The free –COOH group is thought to be responsible for the GI toxicity associated with all traditional NSAIDs. In the present research work, the main motto was to develop new chemical entities as potential anti-inflammatory agents with no GI toxicities. In this paper, the results of synthesis and pharmacological screening of a series of S-substituted phenacyl 1,3,4-oxadiazoles and Schiff bases derived from 2-[(2,6-dichloroanilino) phenyl] acetic acid (diclofenac acid) are described. The 1,3,4-oxadiazoles and diclofenac moieties are important because of their versatile biological actions. In the present studies, the oxadiazole system has been functionalized onto the diclofenac acid moiety and 18 compounds in this series were synthesized. The structures of new compounds are characterized by TLC, FTIR, ^{1}H NMR and Mass spectral data. These compounds were tested in vivo for their anti-inflammatory activity. The compounds, which showed significant activity (comparable to the standard drug diclofenac sodium), were screened for their analgesic activity and to check their ability to induce ulcers by ulcerogenicity and histopathology studies. Eight new compounds, out of 18, were found to have significant anti-inflammatory activity in the carrageenan induced rat paw oedema model, with significant analgesic activity in the acetic acid induced writhing model with no ulcerogenicity. The compounds, which showed negligible ulcerogenic action, also showed promising results in histopathology studies, that is, they were found to be causing no mucosal injury.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain and inflammation, particularly for different types of arthritis.[1–3] Among the most popular NSAIDs worth mentioning is diclofenac sodium, which is approved in more than 120 countries across the globe since its introduction, 28 years ago, and is ranked 30th among the top 200 drugs with respect to new prescriptions.[4]

The pharmacological activity of NSAIDs is related to the suppression of prostaglandin biosynthesis from arachidonic acid by inhibiting the enzyme prostaglandin endoperoxidase, popularly known as cyclo-oxygenase (COX).[5,6] It was discovered that COX exists in two isoforms, COX-1 and COX-2, which are regulated and expressed differently.[7–9] COX-1 provides cytoprotection in the gastrointestinal tract (GIT), whereas inducible COX-2 selectively mediates inflammatory signals.[10–12] Since most of the currently available NSAIDs in the market show greater selectivity for COX-1 than COX-2,[13] chronic use of NSAIDs, including diclofenac, may elicit appreciable GI irritation, bleeding and ulceration.[14] The incidences of clinically significant GI side effects due to long term use of NSAIDs are very high (30%) and cause some patients to abandon NSAID therapy.[4] GI damage from NSAID is generally attributed to two factors. Local irritation by the direct contact of carboxylic acid (–COOH) moiety of NSAID with GI mucosal cells (topical effect) and decreased tissue prostaglandin production in tissues which undermines the

Keywords: Diclofenac; 1,3,4-Oxadiazole; Anti-inflammatory; Analgesic; Ulcerogenicity.
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physiological role of cytoprotective prostaglandins in maintaining GI health and homoeostasis.[5,15]

Synthetic approaches based upon chemical modification of NSAIDs have been taken with the aim of improving safety profile and in turn therapeutic window of this NSAID. Several studies have described the derivatization of the carboxylate function[16–18] of representative NSAID with less acidic azoles, viz. 1,3,4-oxadiazole, Triazole, etc. which resulted in an increased anti-inflammatory activity with reduced ulcerogenicity. Furthermore, it has been reported in the literature that certain compounds bearing 1,3,4-oxadiazole nucleus possess significant anti-inflammatory activity.[19–23] In our attempt to discover new, safer and potent agents for treatment of inflammatory diseases, we have replaced the carboxylic acid group of diclofenac acid with less acidic heterocycle, 1,3,4-oxadiazole, in order to accentuate potency and reduce GI toxicities associated with the parent diclofenac due to its free –COOH group. The compounds designed so were found to possess much significant analgesic-anti-inflammatory profile with significant reduction in potential for ulcerogenic toxicities.[24,25]

2. Results and discussion

2.1. Chemistry

The synthetic route used to synthesize title compounds is outlined in Scheme 1. Ethyl-[2-(2,6-dichloroanilino)-phenyl]acetate (**2**), the starting material, was prepared according to the method reported in the literature, using 2-[(2,6-dichloroanilino) phenyl]acetic acid (diclofenac acid). The acid hydrazide (**3**) was prepared by esterification of 2-[(2,6-dichloroanilino) phenyl]acetic acid (**1**) followed by treatment with hydrazine hydrate in absolute ethanol. The reaction of hydrazide **3** with carbon disulfide in an alkaline medium afforded 5-[2-(2,6-dichloroanilino) benzyl] 2-mercapto-1,3,4-oxadiazole (**4**). Various S-substituted phenacyl-1,3,4-oxadiazoles (**4a**–**h**) were prepared by treatment of hydrazide with various substituted acetophenones in the presence of pyridine. [2-(2,6-dichloroanilino) benzyl carbazide] (**3**) was condensed with different substituted aromatic aldehydes in ethanol to give corresponding Schiff bases (**3a**–**k**) in very good yields. The structures of various synthesized compounds were assigned on the basis of different chromatographic and spectral studies. The physical data, FTIR, ^{1}H NMR and Mass spectral data for all the synthesized compounds are reported in experimental protocols.

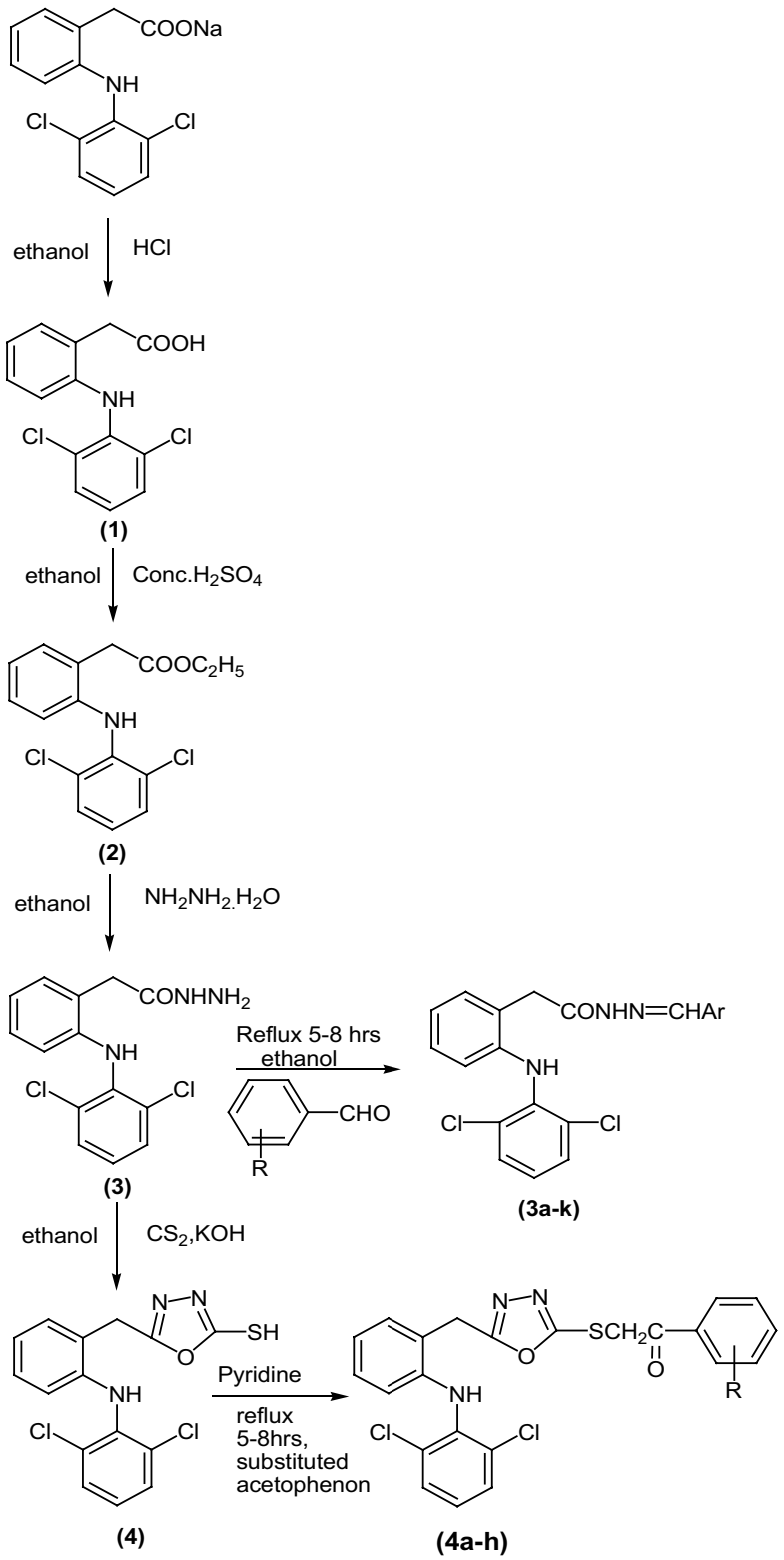
The FTIR spectra of the Schiff bases exhibited very similar features and showed the expected bands for the characteristic groups which are present in the compounds such as C–H and the C=N stretching vibrations and another specific band for Ar–C–N vibrations. Compounds (**3a**–**k**) have C=O stretching bands in the range 1703–1677. For 1,3,4-oxadiazole derivatives, the presence of C=N stretching band at 1666–1598 cm^{-1} is an evidence of ring closure. The schiff bases (**3a**–**k**) and oxadiazole (**4a**–**h**) showed N–H stretching bands at 3362–3236 and 3467–3106 cm^{-1}, respectively. In the proton NMR spectral data, all protons were seen according to the expected chemical shift and integral values. The aromatic protons appeared as multiplet peaks within the range 6.8–7.8 δ ppm, singlet signals derived from hydrazide (–NH–NH_2) structure appeared at 4.23 ppm. Ethylene protons resonated as singlet at 4.86 ppm. The ^{1}H NMR spectra of compounds (**3a**–**k**) and (**4a**–**h**) displayed singlet due to –NH– groups around 8.1, 9.4 and 10.3 ppm (probably due to their ability to get exchanged with D_2O) each signal showing integration for one proton. For the compounds (**3a**–**k**), the signals belonging to benzylidene group were observed at aromatic region, while the signals belonging to –$NHNH_2$ disappeared indicating functionalization of hydrazide to hydrazone with substituted aromatic aldehydes.

2.2. Pharmacology

Continuing our studies on 2-[(2,6-dichloroanilino) phenyl] acetic acid derivatives that are attractive candidates as antinociceptive agents, we have designed a new series of 2-[(2,6-dichloroanilino) phenyl] acetic acids functionalized with different oxadiazole derivatives. In the pharmacological study, we have investigated anti-inflammatory and analgesic activity as well as the acute ulcerogenicity of both oxadiazole derivatives (**4a**–**h**) and Schiff bases (**3a**–**k**). Diclofenac, the parent compound, was used as a reference standard. The experiments were performed on albino rats of Wistar strain of either sex, weighing 100–120 g. The animals were maintained at 25 ± 2 °C, 50 ± 5% relative humidity and 12 h light/dark cycle. The animals were fasted for 24 h prior to the experiments and water provided ad libitum. The test compounds were suspended in 1% aqueous carboxy methyl cellulose (CMC) solution and administered orally to experimental animals.

2.2.1. Anti-inflammatory activity. Anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan induced rat paw oedema model, equimolar doses, that is, equivalent to 0.033 M, of diclofenac acid sub planter injection of 0.1 mL, 1% carrageenan produced increase in paw volume (oedema) of all the animals of various groups. The onset of action was evident from 1 h in various test groups. The significant ($p < 0.01$) reduction of rat paw oedema was observed by most of the test compounds at 3 h compared to control group (Tables 1 and 2). Compounds with significant anti-inflammatory profile were subjected to ulcerogenicity potential test at 12 times the therapeutic doses with additional physical (cold) stress. A thorough examination of the results of histopathological studies indicated absence of the disruption of gastric epithelial morphology and absence of ulcers/erosion in test group animals compared to reference standard, diclofenac acid, and control group animals. The results of the ulcerogenicity studies are presented in Table 4 and results of histopathological studies are depicted in Figure 1a–e.

From close inspection of the results of in vivo experiments, we can conclude that the cyclization of hydrazide moiety to oxadiazole yielded compounds with different



Scheme 1.

therapeutic efficacy. In this series compounds **4b**, **4c**, **4e**, and **4g** exhibited very significant anti- inflammatory activity compared to standard drug diclofenac. The results of two different series of compounds, viz. 1,3,4-oxadiazole derivatives containing substituted acetophenones (**4a**–**h**) and acyclic hydrazones containing substituted aromatic aldehydes (**3a**–**k**) indicate that latter series of compounds are more potent.

Based on findings of these preclinical results, further studies need to be carried out to investigate the other specifications, such as in vitro assays, chronic ulceroge-

Table 1. Results of anti-inflammatory activity of Schiff bases (**3a**–**k**) against carrageenan induced rat paw oedema model in rats

Compound	Change in paw volume in (mL) after drug treatment (±SEM)			Anti-inflammatory activity (% inhibition)		
	1h	2h	3h	1h	2h	3h
Control	1.77 ± 0.08	1.94 ± 0.07	2.31 ± 0.05	—	—	—
Diclofenac	1.97 ± 0.05	2.27 ± 0.04	2.57 ± 0.04	28.69**	48.99**	71.30**
3a	1.84 ± 0.06	2.15 ± 0.05	2.14 ± 0.05	32.28**	48.81**	72.76**
3b	2.02 ± 0.06	2.33 ± 0.06	2.61 ± 0.06	31.38**	46.96**	72.88**
3c	1.98 ± 0.03	2.29 ± 0.04	2.58 ± 0.04	29.11**	50.97**	71.93**
3d	1.67 ± 0.02	1.97 ± 0.02	2.26 ± 0.02	27.21**	51.88**	73.36**
3e	1.82 ± 0.01	2.18 ± 0.00	2.50 ± 0.01	20.87**	47.02**	69.79**
3f	1.48 ± 0.03	1.84 ± 0.03	2.16 ± 0.02	16.64ns	41.35**	60.34**
3g	1.81 ± 0.01	1.95 ± 0.02	2.66 ± 0.01	16.28*	38.14**	70.94**
3h	1.94 ± 0.05	2.28 ± 0.05	2.62 ± 0.04	20.99*	43.09**	68.62**
3i	1.62 ± 0.03	2.00 ± 0.03	2.33 ± 0.05	13.54ns	40.20**	60.99**
3j	1.68 ± 0.02	1.98 ± 0.02	2.28 ± 0.02	28.20**	48.75**	69.39**
3k	2.01 ± 0.02	2.32 ± 0.01	2.65 ± 0.01	30.53**	51.72**	80.07**

Data analyzed by one way ANOVA followed by Dunnett's '*t*' test, ($n = 6$), $^{*}P < 0.05$, $^{**}P < 0.01$ significant from control; ns, not significant.

Table 2. Reults of anti-inflammatory activity of oxadiazole derivatives (**4a**–**h**) against carrageenan induced rat paw oedema model in rats

Compound	Change in paw volume in (mL) after drug treatment (±SEM)			Anti-inflammatory activity (% inhibition)		
	1h	2h	3h	1h	2h	3h
Control	1.77 ± 0.08	1.94 ± 0.07	2.31 ± 0.05	—	—	—
Diclofenac	1.97 ± 0.05	2.27 ± 0.04	2.57 ± 0.04	28.69**	48.99**	71.3**
4a	1.53 ± 0.05	1.91 ± 0.06	2.22 ± 0.03	20.99*	45.64**	67.19**
4b	1.71 ± 0.05	2.10 ± 0.05	2.39 ± 0.00	23.80**	52.09**	74.23**
4c	1.92 ± 0.01	2.27 ± 0.01	2.59 ± 0.01	23.43**	48.63**	72.63**
4d	1.79 ± 0.02	2.09 ± 0.01	2.37 ± 0.02	30.46**	52.26**	60.39**
4e	2.08 ± 0.03	2.34 ± 0.01	2.67 ± 0.03	31.55**	48.91**	71.99**
4f	2.04 ± 0.03	2.35 ± 0.01	2.65 ± 0.03	28.34**	49.47**	60.39**
4g	2.12 ± 0.05	1.91 ± 0.06	2.76 ± 0.04	26.02**	45.16**	71.05**
4h	1.86 ± 0.05	2.23 ± 0.03	2.56 ± 0.04	17.94ns	44.53**	69.89**

Data analyzed by one way ANOVA followed by Dunnett's '*t*' test, ($n = 6$), $^{*}P < 0.05$, $^{**}P < 0.01$ significant from control; ns, not significant.

nicity studies, toxicological studies and mechanism by which these drugs exhibit potential analgesic, anti-inflammatory activity.

2.2.2. Analgesic activity. The analgesic activities of the compounds were studied by using acetic acid induced writhing test in mice. The compounds, which exhibited significant anti-inflammatory activity comparable to that of diclofenac acid, were screened for analgesic activity. The analgesic activity was evaluated at equimolar doses equivalent to **10** mg/kg (diclofenac acid) body weight. These compounds presented an important analgesic profile measured by the classical acetic acid induced writhing model. From the results of acetic acid induced writhing test, it was noticed that all compounds possess significant analgesic activity (Table 3). The analgesic effects of (**3k**) (68.66%) and (**4b**) (66.89%) were found to be better than that of diclofenac sodium (64.65%).

Additionally, these studies showed that the most potent analgesic agent from hydrazone series was (**3k**). While (**4b**) was found to be the most active in the oxadiazole series. When compared all 8 compounds together, the most of the 1,3,4-oxadiazole/hydrazone derivatives were found to have significant analgesic action.

2.2.3. Acute ulcerogenicity studies. Ulcerogenic effect of Schiff bases (**3b**, **3d**, **3k**) and oxadiazole derivatives (**4b**, **4c**) with best overall profile in animal efficacy model was evaluated for gastric ulcerogenic potential in rat stress model at 12 times the therapeutic doses (Table 4). When compared with diclofenac acid, these four compounds did not cause any gastric ulceration and disruption of gastric epithelial cells at the above-mentioned oral doses. Hence gastric tolerance to these compounds was better than that of diclofenac acid indicating that the functionalization of the acidic –COOH group of the diclofenac acid with substituted 1,3,4-oxadiazole and substituted hydrazones has resulted in more potent and nontoxic New Chemical Entities (NCEs). All above results have proved that the rationale for selecting less acidic 1,3,4-oxadiazoles and hydrazones was accurate and fruitful. The results are shown in Table 4.

2.2.4. Histopathological studies. The stomach specimen of diclofenac acid treated rats was characterized by complete disruption of protective mucosal layer (Fig. 1b). Histopathological analysis also showed characteristic features of ulceration in diclofenac acid treated group of animals. The tissue of diclofenac acid treated rats

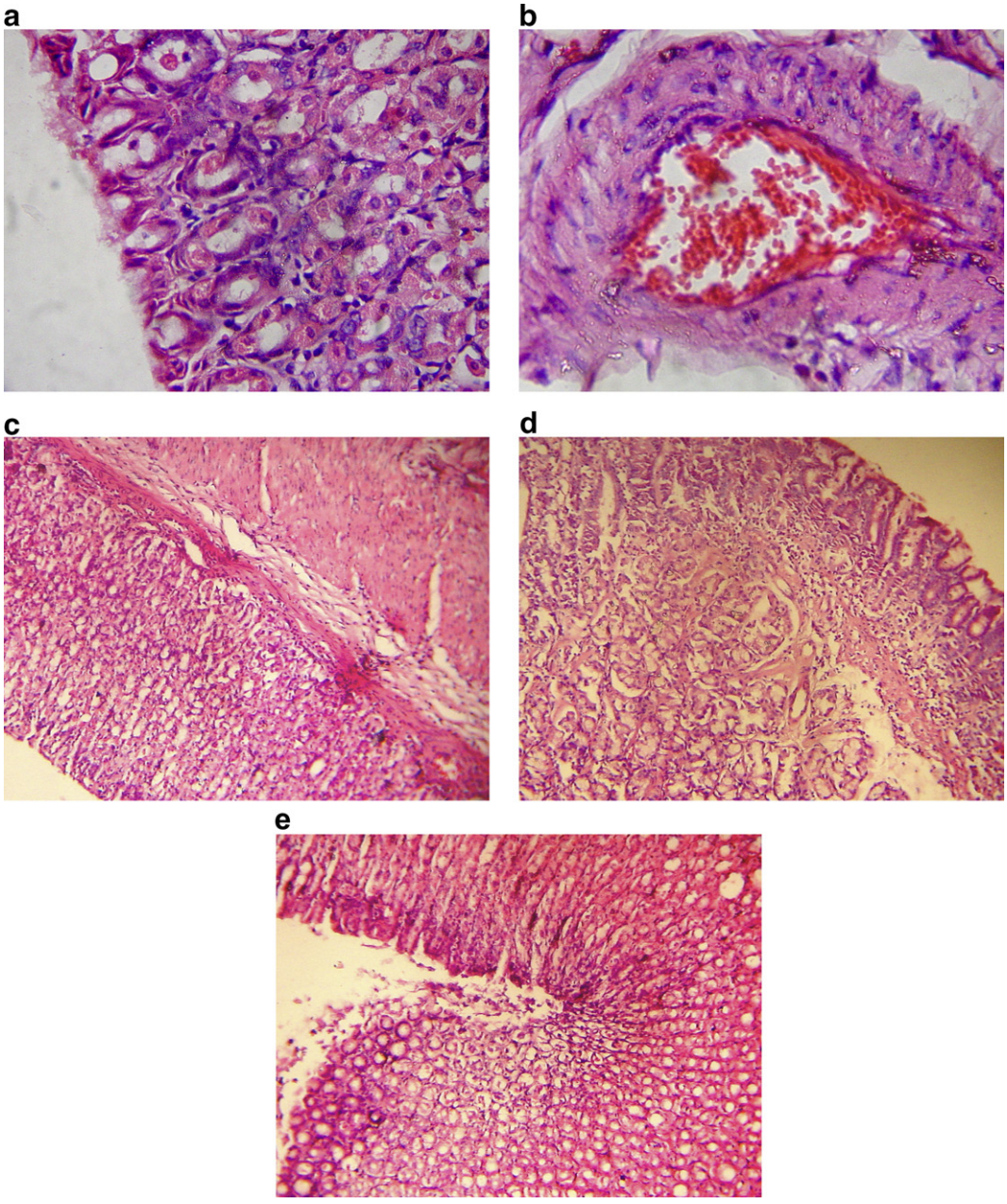


Figure 1. Haematoxylin and eosin Immunohistochemical staining of gastric ulcers after ulcer induction in rats. As illustrated in Fig. specimen (**a**) shows Intact Mucous membrane in control treated rat showing granular tissues composed of macrophages, fibroblasts and endothelial cells forming microvessels. Congestion of mucosal blood vessels in diclofenac treated group, specimen (**b**). No damage was seen to mucosa of rat treated with test compound, **3d**, specimen (**c**), **3k**, specimen (**d**) and **4b**, specimen (**e**), these specimens **c**–**e** were identical to that of the control, specimen (**a**). Original magnification 200×.

has shown that some epithelial cells in the ulcer margin had proliferated and migrated over and into the ulcer crater, which was strongly infiltrated by inflammatory cells, fibroblasts and endothelial cells indicating complete disruption of gastric epithelial layer. Scanning of stomach specimens using electron microscope revealed that in the rats treated with Schiff bases (**3b**, **3d**, **3k**) and oxadiazole derivatives (**4b**, **4c**) there was no injury observed in stomach mucosa. As illustrated in Figure 1, specimen **C** which is identical to that of the control (Fig. 1a).

3. Conclusions

Various substituted oxadiazole and benzylidene hydrazone derivatives were synthesized and screened for analgesic, anti-inflammatory and ulcerogenic potential. Most compounds exhibited significant analgesic and anti-inflammatory activity. Compounds (**3k**) and (**4b**) which have benzylidene hydrazine and oxadiazole moiety showed strong analgesia in acetic acid induced writhing tests. Among all the synthesized compounds, compounds (**3k**) and (**4b**) exhibited most prominent

Table 3. Results of analgesic activity of synthesized compounds against acetic acid induced writhing tests in mice

Compound	Dose (mg/kg, p.o)	No of writhes in 25 min after treatment (Mean ± SEM)	% inhibition
Control	—	27.83 ± 0.477**	—
Std.	10	9.83 ± 0.60**	64.65
3a	10	15.16 ± 0.83**	45.27
3b	10	11.0 ± 0.57**	60.46
3d	10	12.83 ± 1.13**	53.86
3k	10	8.66 ± 0.55**	68.66
4b	10	9.16 ± 0.70**	66.89
4c	10	13.5 ± 0.99**	51.36
4e	10	8.83 ± 0.47**	68.22
4g	10	12.5 ± 0.76**	55.00

Data analyzed by one way ANOVA followed by Dunnett's '*t*' test, ($n = 6$), $^{**}P < 0.01$ significant from control.

Table 4. Ulcerogenic effects of synthesized compounds in comparison with diclofenac sodium

Compound	Dose (mg/kg, p.o)	Ratio of ulcerated animals	Ulcer index (mean ± SE)
Diclofenac	10	4/6	1.8 ± 0.1
	30	6/6	2.1 ± 0.2
	50	Not tested	—
3b	10	—	—
	30	—	—
	50	—	—
3d	10	—	—
	30	—	—
	50	—	—
3k	10	—	—
	20	—	—
	50	—	—
4b	10	—	—
	30	—	—
	50	—	—
4c	10	—	—
	30	—	—
	50	—	—

and consistent anti-inflammatory activity. From the detailed analysis of the results of histopathological studies, we conclude that the synthesized compounds have not only retained the anti-inflammatory profile of diclofenac acid but also have helped in enhancing the anti-inflammatory activity and are devoid of the deadlier gastrointestinal toxicities.

4. Experimental

Melting points were determined by open capillary tubes and are uncorrected. FTIR spectra of the powdered compounds were recorded using KBr on a Jasco FTIR V 430+ spectrometer using Diffuse Reflectance Attachment and are reported in cm^{-1} and ^1H NMR spectra were recorded on a Varian Mercury YH300 (300 MHz FT NMR) spectrophotometer using TMS as an internal reference (Chemical shift represented in δ ppm). Mass spectra were recorded on GC-MS QP5050ASystem (benchtop quadrupole mass spectrophotometer). Purity of the compounds was checked on TLC plates using silica gel G as stationary phase and iodine vapors as visualizing agent. The 2-[(2,6-dichloroanilino) phenyl] acetic acid sodium salt (diclofenac sodium) was procured from Iatros Pharmaceuticals Ltd., Pune, India.

4.1. Hydrolysis of diclofenac sodium to 2-[(2,6-dichloroanilino) phenyl] acetic acid (1)

Diclofenac sodium (0.101 mol) was dissolved in ethanol (2.5 mol); to this solution conc.H_2SO_4 was added dropwise to hydrolyse the salt to acid. The acid obtained was filtered, dried mp 153–155 °C.

4.2. Synthesis of ethyl-[2-(2,6-dichloroanilino) phenyl] acetate (2)

The ethyl ester was prepared as per procedure reported in the literature.[26]

4.3. Synthesis of [2-(2,6-dichloroanilino) phenyl] acetic acid hydrazide (3)

The hydrazide was prepared as per the reported procedure,[27] yield: 72%; mp 134–136 °C. FTIR spectra of the compound showed bands at 3325 (N–H); 2970 (C–H); 1638 (C=O). ^1H NMR chemical shifts at ($CDCl_3$, δ ppm): 3.59 (s, 2 H, CH_2CO); 4.14 (s, 2H, NH_2); 6.82–6.98 (m, 4H, 3,4,5,6 ArH); 7.21–7.27 (m, 3H, dichloro ArH); 7.54 (s, 1H, NH); 8.27 (s, 1H, CONH). Mass spectra of compound exhibited molecular ion peak at *m*/*z* 309 (M^+), other important fragments were observed at 310 (M^++1), 311 (M^++2), 313 (M^++4), 278, 250, 214.

4.4. General procedure for the preparation of Schiff bases (3a–k)

The Schiff bases were prepared as per the reported method[28] and purified by recrystallization from DMSO.

4.4.1. *N*-(Cinnamaldenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3a). Mp 200–202 °C; yield 87%; FTIR (KBr) cm^{-1}: 3285 (NH), 1677 (C=O), 1598 (C=N), C=C, 747 (C–Cl); ^1H NMR; mass: *m*/*z* 424 (M^+), 423 (26%), 425 (M+1) (64.6%); ^1H NMR (300 MHz, DMSO-d_6), 8.6 (s, 1H, CH=N), 7.22–7.29 (m, 4H, 3,4,5,6 ArH), 7.45–7.48 (m, 3H, dichloro ArH), 7.35 (m, 3H, phenyl ring),11.87 (bs, 1H, –CONH–), 8.1 (s, 1H, –NH–).

4.4.2. *N*-(2-Hydroxybenzylidenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3b). Mp 210–212 °C ; yield 70%; FTIR (KBr) cm^{-1}: 3285 (NH), 1685 (C=O), 1624 (C=N), 3293 (OH), 752 (C–Cl); mass: *m*/*z* 413 (M^+), 414 (M+1), 415 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.5 (s, 1H, CH=N), 13.0 (s, 1H, OH), 7.45–7.48 (m, 3H, dichloro ArH), 7.31–7.36 (m, 4H, phenyl ring), 7.22–7.29 (m, 4H, 3,4,5,6 ArH), 10.17 (bs, 1H,–CONH–), 9.4 (s, 1H, NH).

4.4.3. *N*-(3,4,5-Trimethoxybenzylidenyl)-[2-(2,6-dichloroanilino) benzyl carbazide] (3c). Mp 218–220 °C; yield 65%; FTIR (KBr) cm^{-1}: 3262 (NH), 1689 (C=O), 1577 (C=N), 1166 (OCH_3), 782 (C–Cl); mass: *m/z* 487(M^+), 488 (M+1), 489 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.9 (s, 1H, CH=N), 7.45–7.48 (m, 3H, dichloro ArH), 7.31–07.36 (s, 2H, phenyl ring), 7.22–7.29 (m, 4H, 3,4,5,6 ArH), 3.91 (s, 3H, *p*-OCH_3), 3.86–3.88 (s, 6H, both *m*-OCH_3), 1.58 (s, 2H, CH_2), 11.21 (bs, 1H, –CONH–),10.3 (s, 1H, NH).

4.4.4. *N*-(4-Methoxybenzylidenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3d). Mp 230–232 °C; yield 78%; FTIR (KBr) cm^{-1}: 3259 (NH), 1701 (C=O), 1600 (C=N), 1164 (–OCH_3), 782 (C–Cl); mass: *m/z* 427(M^+), 428 (M+1), 429 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.14 (s, 1H, CH=N), 7.45–7.48 (m, 3H, dichloro ArH), 7.31–7.36 (m, 4H, phenyl ring of schiff base), 7.22–7.29 (m, 4H, 3,4,5,6 ArH), 3.91 (s, 3H, *p*-OCH_3), 10.05 (bs, 1H, –CONH–),8.1 (s, 1H, NH).

4.4.5. *N*-(3-Nitrobenzylidenyl)-[2-(2,6-dichloroanilino)-benzyl carbazide] (3e). Mp 209–212 °C; yield 88%; FTIR (KBr) cm^{-1}: 3357 (NH), 1680 (C=O), 1564 (C=N), NO_2 1531 (asymm) 1347 (symm), (OCH_3), 735 (C–Cl); mass: *m/z* 442 (M^+), 443 (M+1), 444 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.6 (s, 1H, CH=N), 7.45–7.48 (m, 3H, dichloro ArH), 7.22–7.29 (m, 4H, 3,4,5,6 ArH),11.73 (bs, 1H, CONH), 6.94–7.1 (m, 4H, phenyl ring of Schiff base), 9.4 (s, 1H, NH).

4.4.6. *N*-(4-Fluorobenzylidenyl)-[2-(2,6-dichloroanilino)-benzyl carbazide] (3f). Mp 180–182 °C; yield 86%; FTIR (KBr) cm^{-1}: 3343 (NH), 1690 (C=O), 1602 (C=N), 1231 (C–F), 740 (C–Cl); mass: *m/z* 430 (M^+), 431 (M+1), 432 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.5 (s, 1H, CH=N), 7.49–7.58 (m, 3H, dichloro ArH), 7.20–7.23 (m, 4H, 3,4,5,6 ArH), 6.94–7.8 (m, 4H, phenyl ring of Schiff base), 10.98 (bs, 1H, –CONH–), 10.3 (s, 1H, NH).

4.4.7. *N*-(4-Hydroxybenzylidenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3g). Mp 238–240 °C; yield 87%; FTIR (KBr) cm^{-1}: 3257 (NH), 1681 (C=O), 1603 (C=N), 3293 (OH), 748 (C–Cl); mass: *m/z* 413 M^+, 414 (M+1), 415 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.9 (s, 1H, CH=N), 10.3 (bs, 1H, CONH), 8.10 (s, 1H, NH), 7.49–7.58 (m, 3H, dichloro ArH), 7.20–7.23 (m, 4H, 3,4,5,6 ArH), 6.83 (s, 2H, phenyl ring), 6.79 (s, 2H, phenyl ring), 4.08 (s, 2H, CH_2),13 (s, 1H, OH).

4.4.8. *N*-(4-Methyl benzylidenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3h). Mp 119–121 °C; yield 65%; FTIR (KBr) cm^{-1}: 3257 (NH), 1683 (C=O), 1563 (C=N), 2902 (C–CH_3), 752 (C–Cl); mass: *m/z* 411(M^+), 412 (M+1), 413 (M+2); ^1H NMR (300 MHz,DMSO-d_6), 8.3 (s, 1H, CH=N), 7.20–7.23 (m, 4H, 3,4,5,6 ArH), 7.49–7.58 (m, 3H, dichloro ArH), 11.35 (bs, 1H, –CONH–), 9.4 NH (s, 1H), 6.94–7.16 (m, 4H, phenyl ring).

4.4.9. *N*-(2-Chloro benzylidenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3i). Mp 119–121 °C; yield 65%; FTIR (KBr) cm^{-1}: 3282 (NH), 1694 (C=O), 1590 (C=N), 760 (C–Cl); mass: *m/z* 431 (M^+), 433 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.14 (s, 1H, CH=N), 6.94–7.66 (m, 4H, phenyl ring), 7.20–7.23 (m, 4H, 3,4,5,6 ArH), 7.49–7.58 (m, 3H, dichloro ArH), 10.59 (bs, 1H, –CONH–), 10.3 (s, 1H, NH).

4.4.10. *N*-(4-Dimethylamino benzylidenyl)-[2-(2,6-dichloroanilino) benzyl carbazide] (3j). Mp 119–121 °C; yield 65%; FTIR (KBr) cm^{-1}: 3263 (NH), 1696 (C=O), 1602 (C=N), 763 (C–Cl); 1367, 1454 ($N(CH_3)_2$); mass: *m/z* 440 (M^+), 441 (M+1), 442 (M+2); ^1H NMR (300 MHz, DMSO-d_6), 8.6 (s, 1H, CH=N), 7.57 (s, 2H, phenyl ring), 7.53 (s, 2H, phenyl ring), 7.25–7.39 (m, 3H, dichloro ArH), 7.0–7.08 (m, 4H, 3,4,5,6 ArH), 3.74 (s, 2H, CH_2), 10.42 (bs, 1H, –CONH–), 8.1 (s, 1H, NH), 3.25 (s, 6H, $N(CH_3)_2$).

4.4.11. *N*-(4-Bromo benzylidenyl)-[2-(2,6-dichloroanilino)benzyl carbazide] (3k). Mp 119–121 °C; yield 65%; FTIR (KBr) cm^{-1}: 3362 (NH), 1666 (C=O), 1621 (C=N), 758 (C–Cl); 556 (C–Br), mass: *m/z* 476 (M^+), 478 (M+2), ^1H NMR (300 MHz, DMSO-d_6); 8.5 (s, 1H, CH=N), 7.39 (m, 3H, dichloro ArH), 7.0–7.08 (m, 4H, 3,4,5,6 ArH), 3.74 (s, 2H, CH_2), 6.94–7.16 (m, 4H, phenyl ring of Schiff base), 11.14 (bs, 1H, –CONH–), 8.1 (s, 1H, NH).

4.5. Synthesis of 5-[2-(2,6-dichloroanilino)benzyl]2-mercapto-1,3,4-oxadiazole (4)

It was prepared as per the reported method.[27] FTIR spectra of the compound showed bands at 3354 (N–H); 1525 (C–N); 1182 (C=S). ^1H NMR ($CDCl_3$, δ ppm): 4.13 (s, 2H, CH_2); 7.25–7.30 (m, 4H, 3,4,5,6 ArH); 7.51–7.53 (m, 3H, dichloro ArH); 7.90 (s, 1H, NH); 10.51 (s, 1H, SH).

4.6. General procedure for the preparation of S-substituted phenacyl 1,3,4-oxadiazole-2-thiol (4a–h)

S-Substituted phenacyl 1,3,4-oxadiazole-2-thiol derivatives were prepared as per the reported procedures.[29]

4.6.1. 5-[2-(2,6-Dichloroanilino) benzyl]-S-(4-nitro phenacyl)-1,3,4-oxadiazole-2-thiol (4a). Mp 110–112 °C; yield 47%; FTIR (KBr) cm^{-1}: 3370(NH), 1675, 1602, 1504, 1450 (ring stretch of oxadiazole nucleus), 1055 (C–O stretch of oxadiazole nucleus), 1345 (symm) 1504 (asymm) NO_2, 790 (C–Cl); mass: *m/z* 514 (M^+), 515 (M+1), 516 (M+2),^1H NMR (300 MHz, $CDCl_3$); 9.4 NH (s, 1H) hump, 4.16 CH_2CO (s, 2H), 6.97–7.11 (m, 4H, 2,3,5,6 ArH), 7.14–7.21 (m, 3H, dichloro ArH), 7.23–7.38 (m, 4H, phenyl ring onto oxadiazole).

4.6.2. 5-[2-(2,6-Dichloroanilino) benzyl]-S-(3-methoxy phenacyl)-1,3,4-oxadiazole-2-thiol (4b). Mp 90–92 °C; yield 71%; FTIR (KBr) cm^{-1}: 3318 (NH), 1577, 1504, 1454 (ring stretch of oxadiazole nucleus), 1065 (C–O stretch of oxadiazole nucleus), 1150 (OCH_3), 779 (C–Cl); mass: *m/z* 499 (M^+), 500 (M+1), 501 (M+2), ^1H NMR (300 MHz, $CDCl_3$), 4.27 (s, 2H, CH_2CO), 3.93 (s, 2H, CH_2), 7.52 (m, 3H, dichloro ArH), 7.24–7.30

(m, 4H, 2,4,5,6 ArH), 3.9 (s, 3H, OCH_3), 4.43–5.31 NH (bs, 1H) hump.

4.6.3. 5-[2-(2,6-Dichloroanilino) benzyl]-S-(3,4-dimethoxy phenacyl)-1,3,4-oxadiazole-2-thiol (4c). Mp 80–82 °C; yield 55%; FTIR (KBr) cm^{-1}: 3068 (NH), 1577, 1500, 1454 (ring stretch of oxadiazole nucleus), 1057 (C–O stretch of oxadiazole nucleus), 1150 (OCH_3), 779 (C–Cl); mass: *m/z* 529 (M^+), 530 (M+1), 531 (M+2); ^1H NMR (300 MHz, $CDCl_3$), 7.49–7.52 (m, 3H, dichloro ArH), 7.24–7.30 (m, 4H, 3,4,5,6 ArH), 7.11–7.13 (m, 3H, dimethoxy phenyl ring), 4.43–5.31 NH (bs, 1H) hump, 4.15 (s, 2H, CH_2CO), 3.93 (s, 2H, CH_2), 2.67. (s, 2H, OCH_3), 2.68 (s, 2H, OCH_3).

4.6.4. 5-[2-(2,6-Dichloroanilino) benzyl]-S-(3,4-dichloro phenacyl)-1,3,4-oxadiazole-2-thiol (4d). Mp 107–110 °C; yield 73%; FTIR (KBr) cm^{-1}: 3467 (NH), 1577, 1506, 1455 (ring stretch of oxadiazole nucleus), 1057 (C–O stretch of oxadiazole nucleus), 778 (C–Cl); mass: *m/z* (M^+) 538, 540 (M+2), 536, ^1H NMR (300 MHz, $CDCl_3$), 7.52–7.54 (m, 3H, dichloro ArH), 7.33–7.37 (m, 4H, 3,4,5,6 ArH), 7.24–7.3 (m, 3H, phenyl ring), 8.1 (s, 1H, NH), 4.17 (s, 2H, CH_2CO), 3.82 (s, 2H, CH_2).

4.6.5. 5-[2-(2,6-Dichloroanilino)benzyl]-S-(4-hydroxy phenacyl)-1,3,4-oxadiazole-2-thiol (4e). Mp 138–140 °C; yield 76%; FTIR (KBr) cm^{-1}: 3318 (NH), 1577, 1506, 1455 (ring stretch of oxadiazole nucleus), 1066 (C–O stretch of oxadiazole nucleus), 3293 (OH), 778 (C–Cl); mass: *m/z* 485 (M^+), 486 (M+1) (26%), 487 (M+2) (68.5%); ^1H NMR (300 MHz, $CDCl_3$), 9.4 NH (s, 1H) hump, 4.16 CH_2CO (s, 2H), 6.97–7.11 (m, 4H, 3,4,5,6 ArH), 7.14–7.21 (m, 3H, dichloro ArH), 7.23–7.38 (m, 2H, phenyl ring on oxadiazole), 7.41–7.42 (m, 2H, phenyl ring on oxadiazole), 3.49 (s, 2H, CH_2).

4.6.6. 5-[2-(2,6-Dichloroanilino)benzyl]-S-(3-nitro phenacyl)-1,3,4-oxadiazole-2-thiol (4f). Mp 120–122 °C; yield 86%; FTIR (KBr) cm^{-1}: 3318 (NH), 1675, 1588, 1505, 1451 (ring stretch of oxadiazole nucleus), 1055 (C–O stretch of oxadiazole nucleus), 1505 (asymm) 1345 (symm) NO_2, 790 (C–Cl); mass: *m/z* 514 (M^+), 515 (M+1), 516 (M+2); ^1H NMR (300 MHz, $CDCl_3$), 10.5 NH (s, 1H) hump, 7.81–7.83 (m, 4H, phenyl ring), 7.43–7.5 (m, 3H, dichloro ArH), 7.24–7.31 (m, 4H, 2,4,5,6 ArH), 4.16 (s, 2H, CH_2CO), 2.68 (s, 2H, CH_2).

4.6.7. 5-[2-(2,6-Dichloroanilino) benzyl]-S-(4-bromo phenacyl)-1,3,4-oxadiazole-2-thiol (4g). Mp 103–105 °C; yield 67%; FTIR (KBr) cm^{-1}: 3318 (NH), 1577, 1504, 1454 (ring stretch of oxadiazole nucleus), 1066 (C–O stretch of oxadiazole nucleus), 561 (C–Br), 777 (C–Cl); mass: *m/z* 548 (M^+), 550 (M+2), 546, ^1H NMR (300 MHz, $CDCl_3$), 10.3 NH (s, 1H) hump, 7.5 (m, 3H, dichloro ArH), 7.24–7.31 (m, 4H, 2,3,5,6 ArH), 4.19 (s, 2H, CH_2CO), 2.38 (s, 2H, CH_2).

4.6.8. 5-[2-(2,6-Dichloroanilino) benzyl]-S-(3-hydroxy phenacyl)-1,3,4-oxadiazole-2-thiol (4h). Mp 145–147 °C; yield 57%; FTIR (KBr) cm^{-1}: 3318 (NH), 1577, 1588, 1506, 1455 (ring stretch of oxadiazole nucleus), 1063 (C–O stretch of oxadiazole nucleus), 3293 (OH), 774 (C–Cl); mass: *m/z* 485 (M^+), 486 (M+1), 487 (M+2); ^1H NMR (300 MHz, $CDCl_3$), 10.5 NH (s, 1H) hump, 7.5 (m, 3H, dichloro ArH), 7.24–7.31 (m, 4H, 2,4,5,6 ArH), 4.21 (s, 2H, CH_2CO), 3.8 (s, 2H, CH_2), 9.9 (s, 1H, OH).

5. Pharmacology

5.1. Animals

Swiss albino mice of either sex weighing 20–25 g and Wistar rats weighing in the range 100–120 g were obtained from National Institute of Virology, Pune, India. All the animals were housed under standard ambient conditions of temperature (25 ± 2 °C) and relative humidity of 50 ± 5%. A 12:12 h light:dark cycle was maintained. All the animals were allowed to have free access to water and standard palletized laboratory animal diet 24 h prior to pharmacological studies. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of College, Pune, constituted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

5.2. Preparation of test compounds

After suspending the test compounds in 1.0% aqueous solution of sodium carboxymethyl cellulose (CMC), test samples were administered to test animals orally. The positive and negative control group animals received the same experimental handling as those of the test groups except that the drug treatment, control group animals received only appropriate volumes of vehicle and of the reference drug, diclofenac sodium, respectively.

5.3. Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the well-known Carrageenan induced rat paw oedema model of Winter et al.[30] using groups of six animals each. A freshly prepared aqueous suspension of carrageenan (1.0% w/v, 0.1 mL) was injected in the subplanter region of right hind paw of each rat. One group was kept as control and the animals of the other group were pre-treated with the test drugs, 1 h before the carrageenan treatment. The volume was measured before and after carrageenan treatment at the 30 min. interval with the help of digital plethysmometer (Panlab LE 7500).

5.4. Analgesic activity

The analgesic activity was evaluated using the acetic acid induced writhing method.[31]

5.5. Acute ulcerogenicity studies

Acute ulcerogenicity screening was done according to method reported by Cioli et al.[32] The mucosal damage

was examined by means of an electron microscope. For each stomach specimen, the mucosal damage was assessed according to the following scoring system.

Score	Description
0.0	Normal (no injury, bleeding and latent injury).
0.5	Latent injury or widespread bleeding (>2 mm).
1.0	Slight injury (2–3 dotted lines).
2.0	Severe injury (continuous lined injury or 5–6 dotted injuries).
3.0	Very severe injury (several continuous lined injuries).
4.0	Widespread lined injury or widened injury.

The mean score of each treated group minus the mean score of control group was regarded as severity index of gastric mucosal damage. Data are expressed as mean ulcer score ±SEM, data analyzed by one way ANOVA followed by Dunnett's '*t*' test to determine the significance of the difference between the standard group and rats treated with the test compounds. The differences in results were considered significant when P was found to be <0.01.

5.6. Histopathological studies[33–35]

For the histopathological study, rats were sacrificed 4 h after the cold stress and their stomach specimens were removed and put into 10% formalin solution. A longitudinal section of stomach along the greater curvature, which included the ulcer base and both sides of the ulcer margin, was taken and fixed in 10% formalin for 24 h at 4 °C and embedded in white solid paraffin. Morphological examination was performed with Haematoxylin and eosin staining to analyze histological changes and examined under electron microscope.

5.7. Statistical analysis

Data obtained for each set of anti-inflammatory model were expressed as mean of change in paw volume ±SD and analyzed by one-way ANOVA followed by Dunnett's test. Data from acetic acid induced writhing model were expressed as mean of number of writhes ±SEM and analyzed by one way ANOVA followed by Dunnett's '*t*' test. Level of significance was set to $P < 0.05$. All statistical calculations were performed using evaluation version of Graph Pad® Prism 3.0 (USA) statistical software.

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